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Influence of Pesticides on UDP-glucuronosyltransferase in Rat Liver Microsomes

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Abstract: Fifteen pesticides, representatives of different chemical groups, were tested for their inhibitory effect on the glucuronidation of 4-nitrophenol (4-NP) and phenolphthalein (PPh) by rat liver microsomes. Three herbicides (simazine, chlorsulfuron, tribenuron-methyl), two insecticides (dioxacarb, carbaryl) and one fungicide (zineb) significantly decreased the UDP-glucuronosyltransferase (UDPGT) activity. The carbamate insecticide dioxacarb was found to be the most potent inhibitor, at 1 mM concentration suppressing 4-NP-UDPGT activity completely, and reducing by 55% the activity associated with the conjugation of PPh. One millimole simazine and carbaryl affected only 4-NP glucuronidation, while chlorsulfuron and zineb exerted a marked inhibition of both 4-NP and PPh conversion. Concentrations of 0·1 mM carbaryl, dioxacarb and zineb were still inhibitory against 4-NP-UDPGT, with zineb producing 40% inhibition of PPh glucuronidation. As a whole, UDPGT isoforms conjugating PPh were less sensitive to the agrochemicals tested. Kinetic studies with dioxacarb, chlorsulfuron and carbaryl revealed a mixed type of inhibition with respect to the acceptor substrate 4-NP, with apparent K_i values of 70 μM, 120 μM and 160 μM, respectively.

Key words: UDP-glucuronosyltransferase, rat liver, pesticides, 4-nitrophenol, phenolphthalein, inhibition.

1 INTRODUCTION

In recent years, pesticides have been widely used in agriculture and their background levels in the environment have increased. These chemicals have been found in many natural products, in foodstuffs and in drinking water, so that exposure to pesticides has become a serious problem. They could be a source of many biochemical and physiological disturbances in animals and humans. Numerous *in-vivo* and *in-vitro* studies of possible consequences of treating animals, cell cultures or enzyme systems with these substances have been conducted.[1–9] Nevertheless, important toxicological aspects of pesticide effects in animal cells remain unknown. In addition, some pesticides (such as dioxacarb, iodofenphos, tribenuron-methyl) have not been investigated, or very few reports concerning their side-effects are available.

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An enzyme system which might be affected by pesticides, is UDP-glucuronosyltransferase. It is a large multigene family of membrane-bound isoenzymes which catalyse the conjugation of glucuronic acid from UDP-glucuronic acid (UDPGA) to hydroxyl-, carboxyl-, amino- or sulfhydryl-groups of many structurally unrelated compounds, permitting them to be eliminated in a water-soluble form. These isoenzymes are found in all mammalian tissues, having the highest activity in the liver. Glucuronidation is an important process in the metabolism and detoxification of a large variety of xenobiotics and endogenous substances.[10–12] A possible inhibition of the glucuronidation reaction by pesticides, which could have toxicological consequences, has attracted our attention.

In this study, 15 commonly used herbicides, insecticides and fungicides, representatives of different chemical groups (Table 1), were tested as inhibitors of 4-nitrophenol and phenolphthalein glucuronidation in

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TABLE 1
List of Pesticides Tested

Common name	*Chemical name*
Herbicides	
1. Lenacil	3-Cyclohexyl-1,5,6-7-tetrahydrocyclopentapyrimidine-2,4(3H)-dione
2. 2,4-D	2,4-Dichlorophenoxyacetic acid
3. Atrazine	6-Chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine
4. Simazine	6-Chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine
5. Diuron	3-(3,4-dichlorophenyl)-1,1-dimethylurea
6. Chlorsulfuron	1-(2-Chlorophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea
7. Tribenuron-methyl	Methyl 2-[4-methoxy-6-methyl-1,3,5-triazin-2-yl(methyl)carbamoylsulfamoyl]benzoate
Insecticides	
8. DDT	1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane
9. Lindane	(1α,2α,3β,4α,5α,6β)-1,2,3,4,5,6-Hexachlorocyclohexane
10. Trichlorfon	Dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate
11. Iodofenphos	*O*-2,5-dichloro-4-iodophenyl *O*,*O*-dimethyl phosphorothioate
12. Carbaryl	1-Naphthyl methylcarbamate
13. Dioxacarb	2-(1,3-dioxolan-2-yl)phenyl methylcarbamate
Fungicides	
14. Zineb	Zinc ethylenebis(dithiocarbamate)
15. Benomyl	Methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate

rat liver microsomes known to be associated with different UDPGT isoforms.[13]

2 EXPERIMENTAL METHODS

2.1 Pesticides and chemicals

DDT, lindane and trichlorfon were obtained from Sigma Chemical Co, St. Louis, MO, USA; 2,4-D was purchased from Aldrich Chemical Co, Milwaukee, WI, USA; atrazine was from Serva, Heidelberg, Germany and lenacil from Du Pont de Nemours Co, Wilmington, DE, USA. Benomyl was a gift from the University of Harare, Zimbabwe.

Simazine, diuron, chlorsulfuron, tribenuron-methyl, iodofenphos, carbaryl, dioxacarb and zineb were extracted with acetone from their respective commercial formulations, and purified by recrystallization. The chemical purity of the extracts was established by thin-layer chromatography on silica gel plates in chloroform + methanol (4 + 1 by volume) as well as by mass spectroscopy. All other chemicals were purchased from Sigma.

2.2 Preparation of microsomes

Liver microsomes from male Wistar rats were isolated by differential centrifugation (10000*g* for 10 min and 105000*g* for 60 min). The microsomal pellets were suspended in 0·1 M Tris-HCl buffer (pH 7·4) containing 0·25 M sucrose, and stored at −70°C until used.[14] The microsomal protein was determined according to Lowry *et al.*[15] with bovine serum albumin as a standard.

2.3 Enzyme assay

UDPGT activities towards 4-nitrophenol (4-NP) or phenolphthalein (PPh) were assayed as described elsewhere.[16,17] The standard incubation medium in a final volume of 250 μl contained the microsomal fraction (0·12 to 0·20 mg protein), Tris-HCl (0·1 M; pH 7·4) EDTA (40 μM), magnesium chloride (10 mM), UDPGA (2 mM) and 4-NP (500 μM) or PPh (120 μM). Glucuronidation was measured on microsomes activated by the nonionic detergent lubrol-17A. The optimal mass ratio detergent/protein was 0·25. The microsome–detergent mixture was preincubated at 4°C for 30 min. The glucuronidation was started by addition of UDPGA (0·01 M; 50 μl). The enzyme reaction was carried out at 37°C for 10 min. The transferase activity was measured colorimetrically at 405 nm for 4-NP or at 550 nm for PPh. The specific activity was expressed as nmoles of glucuronide formed per min per mg protein. All pesticides tested were colourless and were added at a final concentration of 1 mM, dissolved in water or dimethyl sulfoxide (DMSO). The final concentration of DMSO in the incubation mixture was 20 g litre^{-1}. The control specific activity of UDPGT towards 4-NP was 24·45(±3·09) nmol min^{-1} mg^{-1} for the assay without DMSO, and 23·51(±2·92) nmol min^{-1} mg^{-1} protein in the presence of DMSO. For the glucuronidation of PPh these values were 2·8(±0·2) nmol min^{-1} mg^{-1} and 2·6(±0·3) nmol min^{-1} mg^{-1} respectively.

The apparent K_i values were determined from Dixon plots of the data ($1/v$ versus (I)) varying the inhibitor concentrations between 0·25 and 2 mM.

3 RESULTS AND DISCUSSION

The inhibitory effects of pesticides on UDP-glucuronosyltransferase activities converting 4-nitrophenol or phenolphthalein in rat liver microsomes are presented in Table 2. The results are expressed as percentages of inhibition of enzyme activities measured in the absence of pesticides. Seven herbicides, six insecticides and two fungicides of different chemical nature (Table 1) were tested in our study. Of the herbicides, 2,4-D exhibited a marginal inhibitory activity against 4-NP glucuronidation, while lenacil, an uracil derivative, did not show any effect. As previously reported,[18] neither mutagenic nor cytotoxic effects of lenacil on bacterial or animal cells have been found. The triazine compounds, atrazine and simazine, showed interesting properties. The herbicides have similar chemical structures, differing only by one *N*-methyl group (Table 1). However, a great difference in their activity was observed. At 1 mM concentration, atrazine was inactive against glucuronidation of 4-NP, while simazine inhibited it strongly. Simazine was further tested as an inhibitor of PPh glucuronidation. No effect was registered, which means that the UDPGT isoforms converting 4-NP or PPh, respectively, differ in their sensitivity to this substance. Triazine herbicides are widely used in agriculture, so there is a risk of exposure to these chemicals. Feeding corn containing 1–5 mg simazine kg^{-1} has been found to induce accumulation in the blood of guinea pigs of 3·3–3·5 mg $litre^{-1}$ simazine (0·015 mM).[19] This is, however, far below the inhibitory concentration of this herbicide (1 mM) reported here.

Diuron, a very active phenylurea herbicide, was less inhibitory (35% inhibition) against 4-NP glucuronidation than simazine, exerting the same effect also against PPh conjugation (Table 2).

TABLE 2
Inhibitory Effects of Pesticides on UDPGT Activities in Rat Liver Microsomes

Common name	*Concentration (mM)*	*Inhibition (%)*[a]	
		4-NP-UDPGT	*PPh-UDPGT*
Herbicides			
1. Lenacil	1	0	—
2. 2,4-D	1	12 (±2)	—
3. Atrazine	1	0	—
4. Simazine	0·1	0	—
	1	56 (±6)	0
5. Diuron	1	35 (±7)	36 (±6)
6. Chlorsulfuron	0·1	*[b]	10 (±1)
	1	60 (±9)	50 (±7)
7. Tribenuron-methyl	0·1	13 (±2)	*
	1	51 (±4)	39 (±4)
Insecticides			
8. DDT	1	13 (±2)	16 (±3)
9. Lindane	1	0	—
10. Trichlorfon	1	38 (±3)	0
11. Iodofenphos	0·1	*	—
	1	39 (±6)	25 (±5)
12. Carbaryl	0·1	23 (±4)	—
	1	61 (±8)	0
13. Dioxacarb	0·01	0	—
	0·1	32 (±5)	15 (±3)
	1	95 (±7)	55 (±7)
Fungicides			
14. Zineb	0·01	—	12 (±1)
	0·1	30 (±1)	40 (±2)
	1	50 (±9)	53 (±7)
15. Benomyl	1	14 (±3)	—

[a] Values are mean (±SD) of at least three experiments.
[b] * = Less than 10% inhibition.

Chlorsulfuron and tribenuron-methyl are potent sulfonylurea herbicides. They have complicated chemical structures, including a triazine ring and a phenylsulfonylurea part, i.e. parts resembling the structures of simazine and diuron, respectively (Table 1). Recently, the target site of sulfonylurea herbicides has been shown to be the branched-chain amino acid biosynthetic enzyme acetolactate synthase.[20] Various metabolic consequences of treating plants with chlorsulfuron have been reported,[21,22] but relatively little is known of its mode of action in animal cells.[23] In the present study, 1 mM chlorsulfuron caused marked changes in UDPGT activities, inhibiting both 4-NP and PPh glucuronidation by 60% and 50%, respectively (Table 2). These results suggest a high binding capacity of chlorsulfuron to different UDPGT isoforms. Kinetic studies were carried out by assaying the enzyme activity at different herbicide concentrations simultaneously varying 4-NP concentration. Chlorsulfuron was found to be a mixed-type inhibitor with respect to the acceptor substrate 4-NP, with an apparent K_i value of 120 μM (Fig. 1). Another sulfonylurea herbicide, tribenuron-methyl, at a concentration of 1 mM, was also a potent inhibitor of UDPGT activities in rat liver microsomes, being little less effective than chlorsulfuron. However, lower (0·1 mM) concentrations of chlorsulfuron or tribenuron-methyl were much less inhibitory against the enzyme activities studied.

The six insecticides tested could be classified into three chemical groups: organochlorines (DDT, lindane), organophosphates (trichlorfon, iodofenphos) and carbamates (carbaryl, dioxacarb). The environmentally stable DDT caused only a marginal decrease in UDPGT activities at 1 mM concentration, while the presence of lindane had no influence on the 4-NP glucuronidation in rat liver microsomes. The lack of inhibitory effect of both compounds on the enzymes studied is probably due to the very high hydrophobicity and stability of these chemicals. This could prevent their binding to the enzyme molecule. The organophosphates trichlorfon and iodofenphos affected 4-NP-UDPGT activity to the same extent. (38% and 39% inhibition, respectively) at a concentration of 1 mM, but differed in their effect towards PPh glucuronidation. PPh conjugation was inhibited only by iodofenphos and was not susceptible to trichlorfon.

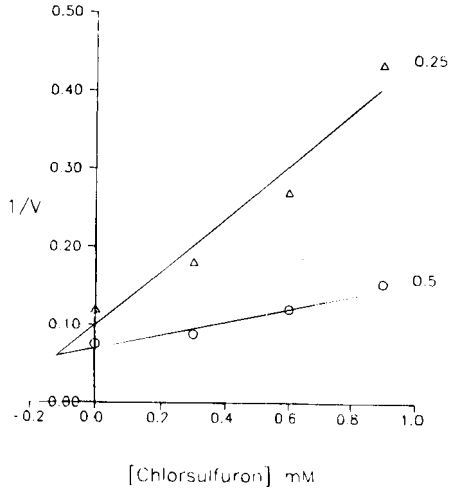

Fig. 1. Dixon plot for the inhibition of 4-NP glucuronidation in rat liver microsomes by chlorsulfuron. Concentrations (mM) of the acceptor substrate 4-NP are indicated on the right-hand-side of the plot. The concentration of UDPGA was kept constant (2 mM).

The representative of carbamates, dioxacarb, proved to be the most potent inhibitor of 4-NP glucuronidation in rat liver microsomes. At 1 mM concentration this insecticide suppressed completely the 4-NP-UDPGT activity and more than 50% of that associated with the conjugation of PPh. Dioxacarb at 0·1 mM was still inhibitory against both enzyme activities, affecting 4-NP glucuronidation the more, while 0·01 mM concentration did not show any effect. Carbaryl was effective only against 4-NP glucuronidation. Further characterization of the interaction of these pesticides with UDPGT isoenzymes converting 4-NP provided the inhibition kinetics. A mixed type of inhibition towards the acceptor substrate 4-nitrophenol was found for both compounds, with apparent K_i values of 70 μM for dioxacarb and 160 μM for carbaryl (Fig. 2). The results suggest that both pesticides compete, at least in part, for the binding site of 4-NP, being, however, capable of interacting also with other parts of the enzyme molecule.

Carbaryl is extensively used in agriculture and for domestic insect control, so the risk of exposure to this chemical is very high. Shilova *et al.*[24] revealed that one month after carbaryl was applied at 5 kg ha^{-1}, the pesticide residues in lemming (*Lemmus sibiricus* L.) and woodcock (*Scolopax rusticula* L.) livers were 1·4 and 1·5 mg kg^{-1}, respectively. In the testes of small animals, carbaryl amounted to 10 mg kg^{-1}. Carbaryl oral LD_{50} values in male and female mice were found to be 108 and 116 mg kg^{-1}, respectively. For dioxacarb these values were lower (77·5 and 61 mg kg^{-1}, respectively).[25] The biochemical effects of carbaryl upon *in-vivo* and *in-vitro* treatment of animal cells have been the subject of numerous studies. Enzyme induction[1,26] or inhibition,[2,27,28] changes of the genetic material as well as of DNA, RNA and protein synthesis,[3] and mutagenesis[29] have been reported. Some of these side-effects might be related to the inhibition of the detoxifying UDPGT. Concerning dioxacarb, a high mutagenic activity against *Saccharomyces cerevisiae* Mayer ex Hansen has been reported.[29] However, very few data on its adverse effects in animal cells are available.[4,5]

Pesticides interfere with the metabolic pathways of target organisms mostly by inhibition of specific enzyme systems. The insecticidal activity of organophosphate and carbamate insecticides is usually correlated with their effect on acetylcholinesterase.[30–32] They inhibit this enzyme by phosphorylation or methyl-

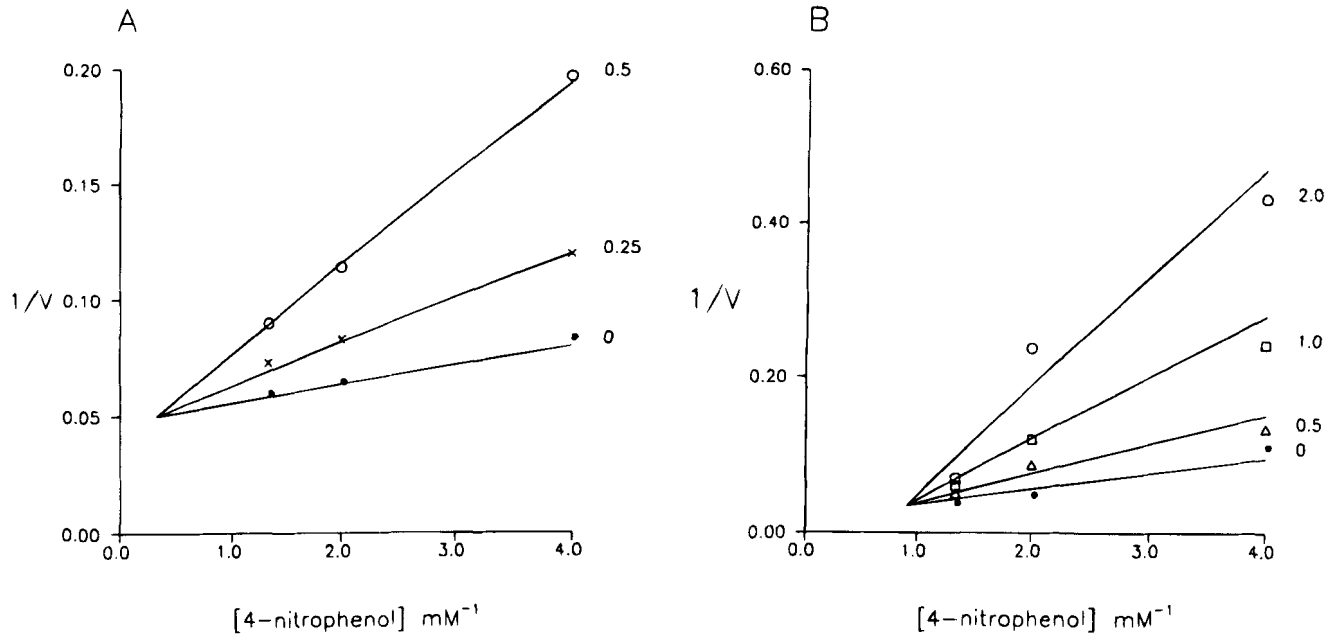

Fig. 2. Lineweaver–Burke plots for the inhibition of 4-NP glucuronidation in rat liver microsomes by (A) dioxacarb and (B) carbaryl. The 4-NP glucuronidation was assayed at varying concentrations of the aglycone in the presence of different concentrations of the insecticide. Insecticide concentrations (mM) are indicated on the right-hand-side of the plots.

carbamoylation, respectively, of the serine residues at the catalytic site. A similar effect with these compounds on acetylcholinesterase of different animal origins has been reported.[1,33] It is possible that such a type of chemical modification may also take place in the molecules of UDPGT in the presence of organophosphate or carbamate insecticides, resulting in enzyme inactivation.

Of the fungicides, the dithiocarbamate derivative, zineb, exhibited a marked inhibitory activity. A concentration of 1 mM of this compound produced 50% inhibition of 4-NP or PPh glucuronidation (Table 2). A significant inhibitory effect upon both enzyme activities, especially towards PPh-UDPGT (40% inhibition), was registered with 0·1 mM zineb and at a concentration as low as 0·01 mM it was still active against PPh conjugation. This fungicide has been also reported to affect other enzymes in animal cells, such as aldehyde dehydrogenase used as a basis of toxicological tests,[7] or the glutathione-dependent enzymes.[6] The genotoxic effects of zineb in rat and mouse liver have also been evaluated.[34] It should be noted that zineb has a very short half-life in the soil.[35]

4 CONCLUSIONS

The results presented in this study show that more than 60% of the tested pesticides exert a direct inhibitory effect on 4-NP-UDPGT activities in rat liver microsomes.

Phenolphthalein glucuronidation is, as a whole, less sensitive to these agrochemicals (with exception of zineb). Dioxacarb, chlorsulfuron and zineb are found to be the most potent inhibitors of UDPGT activities. The inhibition of detoxifying UDPGT isoenzymes could have a number of toxicological consequences. It should be mentioned, however, that 1 mM pesticide concentration producing an *in-vitro* enzyme inhibition exceeds by far that which would be expected in tissues of animals fed with a diet containing pesticide residues. Literature data on the accumulation of the pesticides studied in animal tissues are scarce. On the other hand, dioxacarb and zineb exhibited *in-vitro* inhibitory activity at concentrations far below 1 mM (0·1 mM or even 0·01 mM for zineb). Therefore, an *in-vivo* toxicological relevance of UDPGT inhibition by both pesticides should be considered. The data reported here might be useful in the evaluation of the cytotoxic effects of the pesticides in animal cells.

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